Published in final edited form as:

Nano Lett. 2004 March ; 4(3): 507–511. doi:10.1021/nl035193d.

Chemically Functionalized Carbon Nanotubes as Substrates for Neuronal Growth

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Abstract

We report the use of chemically modified carbon nanotubes as a substrate for cultured neurons. The morphological features of neurons that directly reflect their potential capability in synaptic transmission are characterized. The chemical properties of carbon nanotubes are systematically varied by attaching different functional groups that confer known characteristics to the substrate. By manipulating the charge carried by functionalized carbon nanotubes we are able to control the outgrowth and branching pattern of neuronal processes.

Carbon nanotubes (CNTs) have attracted a great deal of attention due to their unique structural, electrical, and mechanical properties. Recently, there has been an intense interest in exploring some of their novel properties, such as superior strength, flexibility, electrical conductivity, and availability of chemical functionalization,^{1–4} for biological applications both at molecular and cellular levels.

The studies of CNTs interfacing them with biology were mainly focused on the interactions of CNTs and biological molecules, such as DNA,^{5–6} peptides,⁷ and proteins (e.g., streptavidin and feritin).^{8–9} CNTs were found to be biocompatible. ^{10–11} For example, when glucose oxidase molecules were attached to CNTs, they preserved their enzymatic activity. ¹¹ The interactions between biomolecules and CNTs' sidewalls were further explored in a controlled assembly of peptide-coated CNTs into macromolecular structures.⁷ In addition to this peptide-assisted engineering, DNA was used to separate nanotubes based on their conductivity (metallic versus semiconducting CNTs),¹² indicating the use of biological molecules in characterization of nanotubes. This increased understanding of the interactions between CNTs and biological molecules could soon result in further fabrication of biological mechano- and electro-nanodevices and nanosensors.

The studies of interaction between CNTs and living mammalian cells are still limited. Mattson et al.¹³ reported the feasibility of using CNTs as a substrate for neuronal growth. Neurons grown on unmodified multiwalled carbon nanotubes (MWNTs) extended their processes, neurites, while they had more elaborated neurites and branching when grown on MWNTs coated by physisorption of the bioactive molecule, 4-hydroxynonenal. This work suggested the biocompatibility of CNTs as a substrate for neurons. One ramification of this

study is that CNTs could potentially be used for neural prosthesis. Namely, CNTs are not biodegradable, and as such they could be used as implants where long-term extracellular molecular cues for neurite outgrowth are necessary, such as in regeneration after spinal cord or brain injury. There, CNTs could serve as an extracellular scaffold to guide directed neurite outgrowth governed by their tips, growth cones, and also to regulate neurite branching; both of these processes lead to the reestablishment of intricate connections between neurons forming synapses. However, in addition to CNT's biocompatibility and durability, it would be important to demonstrate that the ends of CNTs can be chemically modified in a defined manner to control neurite outgrowth and branching, rather than relying on physisorption, which may exhibit transient retention of attached molecules to the CNTs. To address this issue, in the present study, we chemically modified CNTs by changing their surface charge (Scheme 1). We find that this manipulation at the surface on CNTs can be used to successfully control characteristics of neurite outgrowth.

We produced as-prepared MWNTs (AP-MWNTs) by chemical vapor deposition method, and then terminated the ends with carboxylic groups (MWNT-COOH; Scheme 1).¹⁹ The AP-MWNTs (100 mg) were refluxed in 100 mL of 3 M nitric acid for 24 h to remove the metal catalyst residues in MWNTs samples, to open the end caps of the MWNTs, and to leave them terminated with carboxylic groups. The reported density of surface acidic groups in MWNTs treated with nitric acid is in the range of 0.2–0.5 atomic %.^{17,18} Following this initial modification, we prepared MWNTs in acyl chloride form (MWNT-COCl) as an intermediate product for further chemical functionalization. MWNT-COOH (12 mg) were dispersed in 20 mL of dimethylformamide (DMF) by a 15-minute sonication treatment. Oxalyl chloride (0.4 mL) was added, dropwise, to the dispersion of MWNTs at 0 °C under nitrogen atmosphere. The mixture was stirred at 0 °C for 2 h, and then again at room temperature for additional 2 h. The excess of oxalyl chloride was removed by heating the reaction mixture at 70 °C for 16 h. MWNT-COCl sample was collected by filtration through a membrane (pore size 0.2 µm) and dried under vacuum.

This intermediate product, MWNT-COCl, was further functionalized with either poly-*m*-aminobenzene sulfonic acid (PABS) or ethylenediamine (EN). PABS was synthesized as previously described.¹⁹ MWNT-COCl were reacted in DMF with either PABS or EN at 100 °C for 5 days. The mixture was filtered through membranes (1.2 µm pore-size), and rinsed with ethanol (95%) and then with distilled water, or with DMF and then with ethanol (95%), to remove the excess of PABS or EN, respectively. The black solid of MWNT-PABS or of MWNT-EN were collected on membranes and dried under vacuum overnight. Assuming full conversion of MWNT-COOH, via intermediate MWNT-COCl, into MWNT-EN or MWNT-PABS, the surface charge density of these functionalized MWNTs, originating from protonation/deprotonation, should be in the 0.2–0.5 atomic % range.

We used mid-IR spectroscopy to characterize chemical functional groups that were covalently attached to MWNTs. We prepared semitransparent films for the spectral measurements by spraying CNTs dispersion in ethanol (95%) onto heated ZnSe optical substrates. We obtained measurements using a Nicolet Magna-IR 560 E. S. P. spectrometer. Figure 1 shows typical spectra of modified MWNTs. The peaks at 1640–1645 cm⁻¹ are attributed to the amide carbonyl vibration ($\nu_{\text{C=O amide}}$).

Taken together, we produced three different MWNT modifications that, at physiological pH of 7.35 used to grow neurons, and due to their association constants, will exhibit differential surface charges ranging from negatively charged MWNT-COOH, neutral, zwitterionic MWNT-PABS to positively charged MWNT-EN.

Next, we prepared MWNT films deposited onto polyethyleneimine (PEI)-coated glass coverslips for neuronal growth. The AP-MWNT and chemically functionalized MWNT samples were separately dispersed by sonication in 95% ethanol for 30 min and then sprayed onto preheated (65–75 °C) glass coverslips (round, 12 mm in diameter) that had been precoated with a thin layer of PEI.^{20–22} After the evaporation of ethanol, the MWNTs formed films on the surface of PEI-coated glass coverslips. MWNT-coated glass coverslips were then inlaid into culture dishes (round, 35 mm in diameter) and sterilized under UV light, followed by application of culture medium to the dishes/coverslips prior to their use for neuronal growth. The precoating of glass coverslips with PEI was necessary, because the MWNT films formed on the clean glass coverslips had short retention (minutes to hours) when exposed to aqueous culture medium, resulting in their peeling-off and floating on the culture medium.

Hippocampal neuronal cultures were prepared from 0- to 2-day-old Sprague-Dawley rats using a previously described procedure.²³ Briefly, hippocampal tissue was dissected from the brain and enzymatically treated with papain (20 IU/mL, Sigma) for 1 h at 37 °C, which activity was terminated by incubation with trypsin inhibitor (10 mg/mL, type II-O, Sigma) for 5 min at room temperature. Tissue was triturated using a fire-polished glass pipette, and resulting cellular suspension was plated onto the PEI- or MWNT-coated coverslips. Cultured cells were maintained in a humidified 5% CO₂/95% air incubator at 37 °C in minimum essential medium (MEM, Gibco) supplemented with heat-inactivated fetal bovine serum (10% v/v; Hyclone), Mito+ serum extender (0.1% v/v, Collaborative Biomedical Products), D-glucose (20 mM, Sigma), L-glutamine (2 mM, Gibco), sodium pyruvate (1 mM, Gibco), penicillin (100 IU/mL), streptomycin (100 µg/mL), and sodium bicarbonate (14 mM, Gibco). Mitotic inhibitor, 1-β-D-arabinofuranosyl-cytosine (5 µM; Sigma), was added to cultures after 3 days to suppress the proliferation of nonneuronal cells.

Initially, we examined fixed neurons that we subjected to scanning electron microscopy (SEM) to confirm the attachment of neurons to the MWNTs as previously described (Figure 2A,B).¹³ For SEM analysis, cultured neurons were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The coverslips were washed with 0.1 M sodium cacodylate three times. Neurons were then incubated with 1% osmium tetroxide in 0.1% sodium cacodylate for 1 h at room temperature followed by washing. Samples were dehydrated by the increased concentrations of ethanol (30%, 50%, 70%, 80%, 95%, and 100%), critical-point dried, and then coated with a thin layer of gold/palladium prior to examination and imaging using a Philips SEM XL30 microscope. AP-MWNTs were permissive substrates for neuronal growth (Figure 2A,B). Neurons on nanotubes grew at least one week in culture, indicating that AP-MWNT tubes may support long-term neuronal survival. This is consistent with previous work utilizing fixed neuronal cultures.¹³

The living neurons were studied using differential interference contrast (DIC) and fluorescence microscopy (Figure 2–Figure 5). In these experiments, performed at room temperature (20–22 °C), we examined cells at day 3 in culture by using a microscope (Nikon TE300) equipped with wide-field epifluorescence illumination from a xenon arc lamp (100 W) and a standard FITC/fluorescein filter set (Chroma Technology Corp., Rockingham, VT). Images were captured using a 60 × Plan Apo objective and a CoolSNAP-HQ cooled charge-coupled device (CCD) camera (Roper Scientific Inc., Tucson, AZ) driven by V++ imaging software (Digital Optics Ltd., Auckland, New Zealand). To reduce photo-bleaching, we inserted in the excitation pathway an electronic shutter (Vincent Associates, Rochester, NY) that was controlled by software.

Neurons were identified based on their morphology, and in some experiments we used a neuron-specific tag to confirm their morphological identification. Hence, neurons were

labeled with FITC-conjugated C fragment of tetanus toxin (CFITC, 10 $\mu\text{g/mL}$ for 1 h at 37 $^{\circ}\text{C}$; Cat. No. 196, List Biological Laboratories, Campbell, CA) as previously described (Figure 2C,D).²⁴ We found that all neurons grown on a standard permissive substrate, PEI ($n = 39$), and on AP-MWNTs ($n = 20$), which we morphologically identified as neuronal cell type, also showed positive staining with CFITC (Figure 2C and D). Consequently, in all subsequent experiments we used a morphological identification to study live neurons.

To test neuronal viability in cultures, we incubated neurons grown on PEI (positive control) or MWNTs with acetoxymethyl ester of calcein (1 $\mu\text{g/mL}$, Molecular Probes, Eugene, OR) in the presence of Pluronic F127 (0.025%, Molecular Probes) for 20 min at 37 $^{\circ}\text{C}$.²⁵ After wash we examined neurons under the fluorescent microscope to determine their viability, as indicated by their ability to accumulate a vital stain, calcein. We found that all neurons examined on PEI ($n = 45$) and on MWNTs ($n = 152$) accumulated dye, indicating their viability (Figure 2E,F).

After this initial evaluation of the biocompatibility of MWNTs, we studied permissiveness of AP-MWNTs to neuronal growth as compared to PEI, a known well-permissive substrate for neuronal growth. We found that, although AP-MWNTs are not as permissive a substrate as PEI, they allowed neuronal growth, characterized by the presence of growth cones, neurite outgrowth, and branching (Figure 2C–F and Figure 3). AP-MWNTs reduced the initiation of neurite outgrowth as characterized by the reduction in the numbers of growth cones and neurites per neuron (Figure 3, top), as well as in the neurite branching (Figure 3, bottom). Interestingly, we found no difference in the average neurite length when comparing measurements originating from neurons grown on AP-MWNTs with those grown on PEI (Figure 3, middle; Student's *t*-test, $p > 0.3$), indicating that MWNTs did not affect the length of neurites that succeeded in their outgrowth.

Next we tested whether the surface charge of the MWNTs can modulate the neurite outgrowth and branching. To address this issue we grew neurons on MWNTs that had been chemically modified as outlined in Scheme 1. SEM examination suggests that neurons grew well on any of these substrates (Figure 4A–C). To evaluate a potential modulatory effect of the charges, we imaged live neurons accumulating calcein (Figure 4D–F), and quantify several different parameters regarding the neurite outgrowth (Figure 5). Although we found no difference between the number of neurites per neuron when these cells were grown on differently functionalized MWNT (Figure 5, top; one-way ANOVA, $p > 0.9$), the average length of neurites was longer when the neurons were plated onto positively charged MWNT-EN (conjugate acid at physiological pH), rather than on zwitterionic MWNT-PABS or negatively charged MWNT-COOH (conjugate base) (Figure 5, middle). Previous studies indicated that substrate qualities play a role in the process of growth cone motility and neurite branching.^{13,26} We found that neurons grown on MWNT-PABS or MWNT-EN showed a higher number of growth cones than those neurons grown on MWNT-COOH (Figure 5, top), indicating that negatively charged surfaces are ineffective in promoting the initiation of the growth cones. Branching of neurites showed graded dependency of MWNT charge in the order MWNT-EN > MWNT-PABS > MWNT-COOH (Figure 5, bottom), suggesting that by using chemically modified MWNTs we can control the neurite branching. This control on branching could be attributed to the charge on the different types of chemical functionalities attached to MWNTs. In the culture media (pH = 7.35), the carboxylic groups on MWNT-COOH are deprotonated ($\text{pK}_a \sim 4.5$) resulting in negatively charged MWNT-COOH, while MWNT-EN are positively charged due to their higher pK_a of amine groups ($\text{pK}_a \sim 9.9$). MWNT-PABS, containing both amine and sulfonic groups, are nearly zwitterionic, being electoneutral, at pH 7.35, and, therefore, having similar effects on neurite outgrowth as those seen when neurons were plated on AP-MWNTs (compare Figure 3 and Figure 5).

We systematically varied the chemical functionalization of CNTs as substrates for neuronal growth. The results show that the surface charge of MWNTs can be used to control the neurite outgrowth as characterized by the presence of more numerous growth cones, longer average neurite length, and elaborate neurite branching when we compared measurements for neurons grown on positively charged MWNTs as opposed to neutral or negatively charged MWNTs (Scheme 2). The culturing method where we grew neurons on MWNTs contrasts the most commonly used method in which neurons are grown on a flat substrate that has no resemblance to the cellular surfaces and extracellular matrix (ECM) that neurons encounter in the brain. MWNTs possess diameters (~100 nm) and aspect ratios that are similar to those of small nerve fibers. Thus, chemical functionalization of MWNTs with different molecules will allow us to gain knowledge on the characteristics of cellular and ECM's molecular cues for neurite outgrowth similar to those found in the brain. Further, because one can produce nanotubes in semiconducting and metallic forms, it will become possible to interface nanotubes and neurons for amperometric and electrophysiological analyses of neuronal circuits. The ability to chemically modify MWNTs in order to control the neurite outgrowth could be implemented clinically, especially in the cases in which long-term presence of outgrowth modulation is necessary. This approach using MWNT prosthesis has advantages over presently used growth substrates, because CNTs offer morphology reminiscent of neuronal process, long-term durability, inertness, and functionalization. Hence, they could be used as scaffolding for the formation of functional neuronal circuits. Some of the future investigations will be conducted to assess the establishment of synaptic transmission between neurons grown on patterned and/or chemically modified CNTs as substrates.

Acknowledgments

This work was supported by a grant from DOD/DARPA/DMEA under Award No. DMEA90-02-2-0216 and the Whitehall Foundation (Award No. 2000-05-17). V.P. is an Institute for Complex Adaptive Matter Senior Fellow.

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Figure 1. Mid-IR spectra of chemically functionalized MWNTs. The peaks at 1640–1645 cm⁻¹ in the spectra of MWNT-EN and MWNT-PABS are attributed to the amide carbonyl vibration.

Figure 2.

MWNTs are permissive substrate for neurons. Both polyethyleneimine (PEI; positive control, left column images) and as-prepared MWNTs (AP-MWNTs; right column images) support neuronal viability and permit neurite outgrowth. (A, B) SEM images of neurons grown on PEI (A) and AP-MWNTs (B). (C, D) Morphologically identified living neurons were positively stained with a neuronal marker, FITC-conjugated C-fragment of tetanus toxin. (E, F) Fluorescent images showing live neurons, which accumulate a vital stain, calcein. Arrows indicate growth cones. Scale bar: 20 μm, except 10 μm in B.

Figure 3.

Parameters of neurite outgrowth for neurons grown on PEI or AP-MWNTs. Black bars report on values for the left ordinates, while open bars indicate values for the right ordinates. Bars represent means $\pm$ standard errors. Numbers in parentheses indicate the number of neurons studied in each condition, and grown in three different cell cultures. Asterisks indicate significant difference in measurement when compared to control (PEI; ** $p < 0.01$).

Figure 4.

Chemically functionalized MWNTs provide permissive substrates for neurons. (A–C) SEM images of neurons growing on different chemically functionalized MWNTs. (D–F) Fluorescent images of live neurons, accumulating calcein, which grow on different chemically functionalized MWNTs. Scale bar: 20 μm , except 10 μm in A–B.

Figure 5.

Parameters of neurite outgrowth for neurons grown on chemically functionalized MWNTs. Black bars represent values for left ordinates, while open bars indicate values for right ordinates. Bars represent means $\pm$ standard errors. Numbers in parentheses indicate the number of neurons studied in each condition. Asterisks indicate significant difference in measurements after one-way ANOVA followed by the Fisher's least square difference test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$).

Scheme 1.
Chemical Functionalization of Multiwalled Carbon Nanotubes (MWNTs)

Scheme 2.

Drawing Summarizing the Effects of MWNT Charges on the Neurite Outgrowth and Branching